



Inactivation by Hg^{2+} and methylmercury of the glutamine/amino acid transporter (ASCT2) reconstituted in liposomes: Prediction of the involvement of a CXXC motif by homology modelling

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ARTICLE INFO

Article history:

Received 21 April 2010

Accepted 21 June 2010

Keywords:

Methylmercury
Glutamine
Transport
ASCT2
Membrane
Liposomes

ABSTRACT

The effect of HgCl_2 , methylmercury and mersalyl on the glutamine/amino acid (ASCT2) transporter reconstituted in liposomes has been studied. Mercuric compounds externally added to the proteoliposomes, inhibited the glutamine/glutamine antiport catalyzed by the reconstituted transporter. Similar effects were observed by pre-treating the proteoliposomes with the mercurials and then removing unreacted compounds before the transport assay. The inhibition was reversed by DTE, cysteine and N-acetyl-cysteine but not by S-carboxymethyl-cysteine. The data demonstrated that the inhibition was due to covalent reaction of mercuric compounds with Cys residue(s) of the transporter. The IC_{50} of the transporter for HgCl_2 , methylmercury and mersalyl, were 1.4 ± 0.10 , 2.4 ± 0.16 or $3.1 \pm 0.19 \mu\text{M}$, respectively. Kinetic studies of the inhibition showed that the reagents behaved as non-competitive inhibitor. The presence of glutamine or Na^+ during the incubation of the mercuric compounds with the proteoliposomes did not exerted any protective effect on the inhibition. None of the compounds was transported by the reconstituted transporter. A metal binding motif CXXC has been predicted as possible site of interaction of the mercuric compounds with the transporter on the basis of the homology structural model of ASCT2 obtained using the glutamate transporter homologue from *Pyrococcus horikoshii* as template.

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1. Introduction

Mercury causes toxic effects in several tissues and organs. In the environment, Hg^{2+} is the most common cationic form and methylmercury is the most common organic mercury compound. It is well known that both cationic and organic mercuric compounds react with thiol groups with very high affinity constants. However, several aspects of the molecular mechanisms of mercury toxicity remain to be elucidated [1–6]. Membrane proteins containing Cys residues exposed to the extracellular environment represent important sites of interaction with mercuric compounds. Transport systems, among membrane proteins, are particularly interesting since they both could allow the toxic compounds to enter the cells and could be chemically modified by these compounds with consequent alterations of their function. Indeed, it was reported that some transport systems catalyze the transport of mercuric compounds through biological

membranes [2,7–9]. Furthermore, interaction of mercuric compounds with transport systems of plasma membrane [1,10–15] and also of mitochondrial membrane [16,17] have been reported to cause alterations of function. In several cases the effects and the sites of interaction of mercurials have been characterized ([14–24] and refs. herein). The study of interaction of mercuric reagents with transport systems for glutamine is still at an initial stage, in spite of the essential role of these transporters in cell homeostasis [25]. It was reported that methylmercury modify the permeability to glutamine of cultured astrocytes by an indirect mechanism based on inhibition of expression of the amino acid transporters ASCT2 and SNAT3; however, this mechanism only partially explained the permeability alteration [26]. The rat glutamine/amino acid transporter ASCT2 which is expressed in kidney, intestine and nervous system besides other tissues [25], was reconstituted in liposomes [27,28]. The transporter was inserted unidirectionally in the proteoliposomal membrane, right-side-out with respect to the cell membrane; thus, the properties of the internal side of the reconstituted transporter correspond to those of the intracellular side and vice versa. The studies in the proteoliposome system, which gives the advantage of reducing the interferences and improving the kinetic resolution in respect to intact cell systems, revealed novel functional properties of this transporter. It was found that the reconstituted transporter is

Abbreviations: C_{12}E_8 , octaethylene glycol monododecyl ether; DTE, 1,4-dithioerythritol.

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functionally and kinetically asymmetrical, is regulated by intraliposomal (intracellular) ATP and functions by a simultaneous mechanism of transport. In preliminary experiments it was also found that externally added mersalyl, p-hydroxymercurybenzoate and HgCl_2 inhibited the transporter [27,28]. Thus, the proteoliposome experimental model is suitable for investigating the effects of mercurial compounds on the ASCT2 transporter. The effects of HgCl_2 and methylmercury, together with the prototypical protein reagent mersalyl, on the transporter have been characterized in this work. Some thiolic antioxidant compounds can reverse the effect of the mercuric reagents. The relationships among the inhibition and the predicted structure of the ASCT2 transporter are described.

2. Materials and methods

2.1. Materials

Amberlite XAD-4 and egg yolk phospholipids (3-sn-phosphatidylcholine from egg yolk) were purchased from Fluka; C_{12}E_8 from Anatrace (USA 434W, Dussel Drive Maumee, OH 43537); L-[^3H]glutamine from GE Healthcare (Milano, Italy); Sephadex G-75, L-glutamine, Adenosine 5'-triphosphate, N-acetyl-L-cysteine, L-cysteine, S-carboxymethyl-L-cysteine, methylmercury, HgCl_2 , mersalyl and all the other reagents were from Sigma-Aldrich (Milano, Italy).

2.2. Solubilization of the glutamine/amino acid transporter

Brush-border membranes were prepared from rat kidney [29] and stored as previously described [14]. The glutamine/amino acid transporter was solubilized by treating the membrane preparation (50 μl , about 0.15 mg protein) with 1.3% C_{12}E_8 in a final volume of 150 μl and centrifuged at $13,000 \times g$ for 4 min at 4°C . The supernatant (extract) was used for the reconstitution.

2.3. Reconstitution of the glutamine/amino acid transporter into liposomes

The glutamine/amino acid transporter was reconstituted by removing the detergent with a hydrophobic chromatography column [16,30]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through the same Amberlite XAD-4 column. The composition of the initial mixture used for reconstitution was: 25 μl of the solubilized protein (25–35 μg protein in 1.3% C_{12}E_8), 75 μl of 10% C_{12}E_8 , 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as previously described [30], 30 mM L-glutamine (except where differently specified), 4 mM ATP, 20 mM Hepes/Tris pH 7.0 in a final volume of 700 μl . All the operations were performed at 4°C . After vortexing, this mixture was passed 16 times at room temperature through the same Amberlite column (0.5 cm diameter \times 2.5 cm height) preequilibrated with a buffer of the same composition of the initial mixture with the exception of protein, detergent and phospholipid.

2.4. Transport measurements

To remove the external substrate, 550 μl of proteoliposomes were passed through a Sephadex G-75 column (0.7 cm diameter \times 15 cm height) preequilibrated with 20 mM Hepes/Tris pH 7.0 and sucrose at an appropriate concentration to balance the internal osmolarity (“unlabeled” proteoliposomes). For efflux measurements, the “unlabeled” proteoliposomes (600 μl), containing 30 mM glutamine, were “prelabeled” by transporter-mediated exchange equilibration [16] by incubation with 10 μl of 0.6 mM

[^3H]glutamine at high specific radioactivity (2 $\mu\text{Ci/nmol}$) for 60 min at 25°C ; then, the external radioactivity was removed by passing again the proteoliposomes through Sephadex G-75 as described above. Transport was started, in the case of uptake, by adding [^3H]glutamine at the indicated concentrations and 50 mM Na-gluconate to the “unlabeled” proteoliposomes, or, in the case of efflux, by adding non-radioactive substrates to the “prelabeled” proteoliposomes. In both cases, transport was stopped by adding 20 μM mersalyl at the desired time interval. In control samples the inhibitor was added at time zero according to the inhibitor stop method [31]. The assay temperature was 25°C . Finally, each sample of proteoliposomes (100 μl) was passed through a Sephadex G-75 column (0.6 cm diameter \times 8 cm height) to separate the external from the internal radioactivity. Liposomes were eluted with 1 ml 50 mM NaCl and collected in 4 ml of scintillation mixture, vortexed and counted. For the determination of the specific activity of [^3H]glutamine uptake, the experimental values were corrected by subtracting the respective controls (samples inhibited at time zero); the initial rate of transport was measured by stopping the reaction after 10 min, i.e., within the initial linear range of [^3H]glutamine uptake into the proteoliposomes. [^3H]glutamine efflux activity cannot be calculated as specific activity for methodological reasons [16]. It was expressed as % residual intraliposomal radioactivity with respect to the radioactivity present in the proteoliposomes at time zero.

2.5. Other methods

The protein concentration was determined by the modified Lowry procedure [32].

Mercuric conjugates of cysteine or N-acetylcysteine were formed by incubating HgCl_2 , methylmercury or mersalyl with cysteine or N-acetylcysteine with 1:1.2 ratio as described in Ref. [7]. The homology structural model of the ASCT2 was built using the glutamate transporter homologue from *Pyrococcus horikoshii* crystal structure (1XFH) as the template. The amino acid sequence of the rat ASCT2 (NP_786934) and the glutamate transporter (NP_143181) were aligned using ClustalW and the alignment was manually adjusted as described in Ref. [33] for ASCT1, a member as ASCT2, of the family of glutamate and neutral amino acid transporters. The optimized alignment was used to run the program Modeller 9v7 [34].

3. Results

3.1. Inhibition of the transporter by mercuric reagents

The effect of HgCl_2 , methylmercury and mersalyl on the glutamine/amino acid (ASCT2) transporter reconstituted in liposomes was investigated. The transport activity was measured as 0.1 mM [^3H]glutamine uptake into proteoliposomes containing 30 mM glutamine (glutamine/glutamine homologous antiport), in the absence or presence of externally added mercuric reagents. The time courses of the glutamine transport are shown in Fig. 1. As previously described [27], the accumulation of labeled glutamine into the proteoliposomes depended on the time. It reached the equilibrium at 60 min with a transport activity of 140–170 nmol/mg protein in the different experiments (\circ). The addition during the transport assay of each of the reagents strongly inhibited the transport (\triangle). In the presence of HgCl_2 (Fig. 1A), methylmercury (Fig. 1B) or mersalyl (Fig. 1C) 75, 69 or 43% inhibition at 15 min was found; at 90 min the transport was still inhibited by 67, 47 or 34%. The experimental data was interpolated in a first order rate equation. The initial transport rate, calculated as the product of k and transport at equilibrium, was $11.8 \pm 1.4 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$ in absence of reagents and 1.7 ± 0.7 , 2.3 ± 0.2 or 5.5 ± 0.2 in the presence of HgCl_2 ,

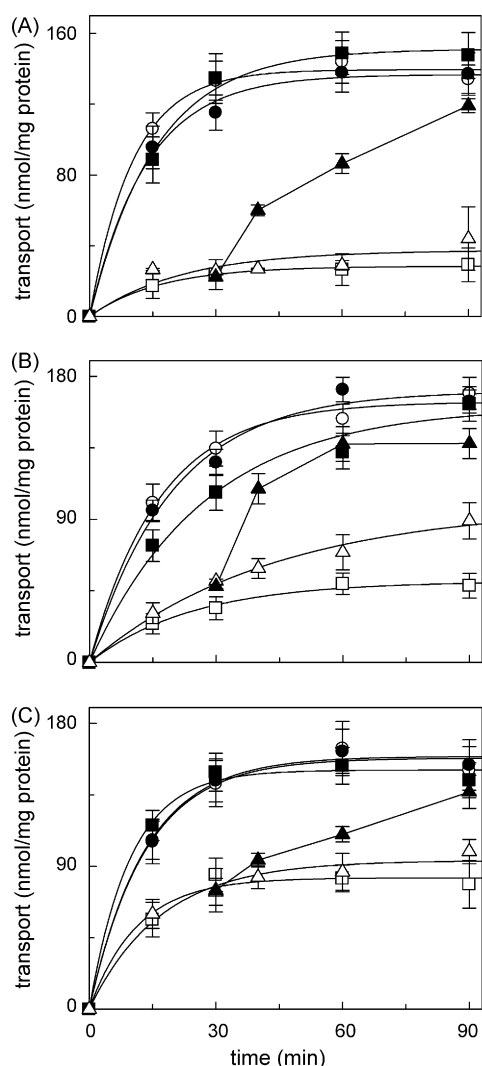


Fig. 1. Effect of mercuric reagents on the glutamine antiporter mediated by the reconstituted transporter. After passage of the reconstituted proteoliposomes through Sephadex G-75 columns, transport was measured adding 0.1 mM [^3H]glutamine and 50 mM Na-gluconate in the presence of: (○), no additions; (●) 2 mM DTE; (△), 3 μM of HgCl_2 (A), methylmercury (B) or mersalyl (C); (▲), 2 mM DTE, added 30 min after the [^3H]glutamine to an aliquot of proteoliposomes. In (□), 6 μM HgCl_2 (A), methylmercury (B) or mersalyl (C) was added 2 min before and in (■) DTE 1 min before the passage of the proteoliposomes through the Sephadex G-75 columns. Transport was started as described above. The transport reaction was stopped at the indicated times, adding 20 μM mersalyl. The values are means \pm S.D. from three experiments.

methylmercury or mersalyl, respectively. No inhibition was observed in the presence of other chloride salts (NaCl or KCl) instead of HgCl_2 , demonstrating the inhibitory effect was due to Hg^{2+} . It is known that mercuric compounds react with Cys residues of proteins by Hg-S covalent bond [35]. To verify if the mercuric reagents reacted with Cys residues of the glutamine/amino acid transporter, the effect of DTE, which reverses the Hg-S linkage, was tested. The addition of DTE (▲) after 30 min, to the proteoliposomes treated with mercurials, led to recovery of the transport function, which was almost complete after 60 min from the addition (90 min of transport) for each of the reagents (Fig. 1A–C). DTE had virtually no effect when added to the control proteoliposomes (●), in the absence of the reagents. To confirm that the inhibition of transport were caused by covalent reaction of the mercuric reagents with the protein, the residual activity of the transporter was determined after pre-incubation of the proteoliposomes with the mercuric reagents and subsequent removal of the unbound compounds. To achieve this objective a strategy previously described for other

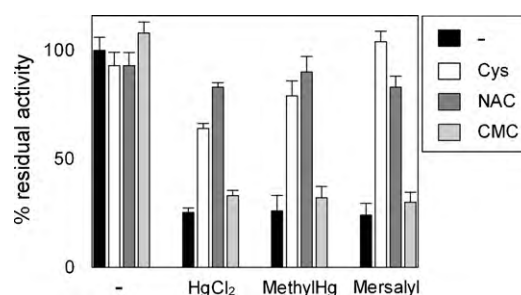


Fig. 2. Effect of cysteine, N-acetylcysteine and S-carboxymethylcysteine on the inhibition of the glutamine antiporter by mercuric reagents. 3 μM HgCl_2 , 6 μM methylmercury or 6 μM mersalyl and, after 1 min, 2 mM cysteine (Cys), N-acetylcysteine (NAC) and S-carboxymethylcysteine (CMC) were added to the reconstituted proteoliposomes, where indicated. After passage of the proteoliposomes through Sephadex G-75 columns transport was measured adding 0.1 mM [^3H]glutamine and 50 mM Na-gluconate and stopped after 30 min, adding 20 μM mersalyl.

transporters [36,37] was adopted. After pre-incubation with each of the reagents, the proteoliposomes were passed through size exclusion Sephadex G-75 columns and, then, the transport activity was measured. Proteoliposomes pre-incubated with the reagents (□) showed inhibition of transport of 84, 76 or 47% at 15 min and 78, 72 or 48% at 90 min, for HgCl_2 (Fig. 1A), methylmercury (Fig. 1B) or mersalyl (Fig. 1C), respectively. Differently, no inhibition was found, in proteoliposomes treated with DTE (■) after the pre-incubation with the reagents and before the passage through Sephadex G-75 (Fig. 1A–C) indicating that all the mercurial-protein linkages had been reversed by DTE. The experiments described clearly showed that the inhibition was caused by covalent interaction of the mercuric reagents with one (or more) SH group of the protein. The effect of cysteine, N-acetylcysteine and S-carboxymethylcysteine on the inhibition was also tested. More than 80% of the transport activity was recovered after incubation of the mercurial treated proteoliposomes with cysteine and N-acetylcysteine except that in the case of HgCl_2 treated proteoliposomes: in this case the recovery of the transport by cysteine was little lower. S-carboxymethylcysteine, which has no free thiol groups, had nearly no effect on the inhibition caused by the mercuric reagents (Fig. 2), confirming the involvement of SH groups of the protein in the reaction. The dependence of the reversal of the inhibition on the concentration of DTE, cysteine and N-acetylcysteine was studied. Maximal effects were observed at concentrations of the thiol containing reagents above 1 mM (not shown).

3.2. Kinetics of the inhibition of the transporter by mercuric reagents

The rate of the glutamine/glutamine antiporter was determined in the presence of increasing concentration of the reagents. The dose–response curves obtained are shown in Fig. 3A. The reagents led to virtually complete inhibition at concentrations above of 3.5, 5 or 40 μM in the case of HgCl_2 , methylmercury or mersalyl, respectively; the IC_{50} values, derived from the dose–response curves, were 1.4 ± 0.10 , 2.4 ± 0.16 or 3.1 ± 0.19 μM , respectively. A similar experiment was performed measuring the antiport rate after pre-incubation of proteoliposomes with the reagents and subsequent removal of the unbound reagents, as described in the second part of the experiment of Fig. 1. The dose–response curves obtained under this condition are reported in Fig. 3B. Results similar to those of Fig. 3A were obtained. The calculated IC_{50} were 2.1 ± 0.40 , 3.0 ± 0.12 or 12 ± 1.7 μM for HgCl_2 , methylmercury or mersalyl, respectively. While in the case of HgCl_2 and methylmercury the IC_{50} values obtained were similar to those calculated for the same reagents added during the transport assay, in the case of mersalyl the IC_{50} value was higher. To get information on the mechanism of inhibition, the dependence of the antiport rate on the extraliposomal glutamine

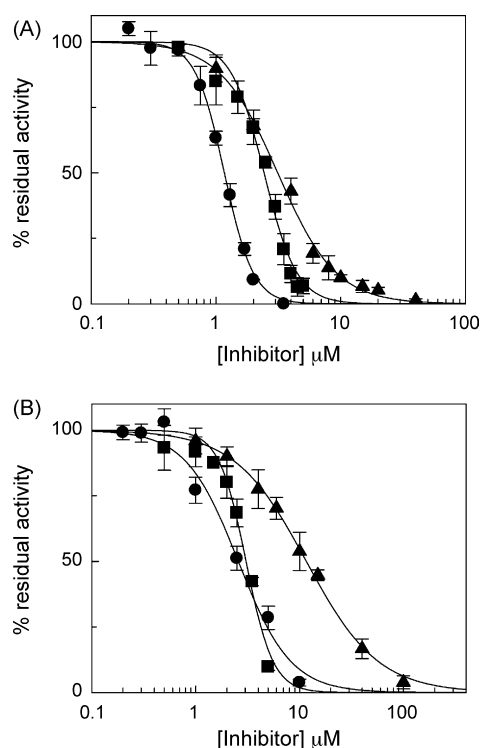


Fig. 3. Dose–response curves for the inhibition of the reconstituted transporter by mercuric reagents. After passage of the reconstituted proteoliposomes through Sephadex G-75 columns, transport was measured adding 0.1 mM [^3H]glutamine and 50 mM Na-gluconate (A) in the presence of HgCl_2 (●), methylmercury (■) or mersalyl (▲) at the indicated concentrations. In (B) HgCl_2 (●), methylmercury (■) or mersalyl (▲) at indicated concentrations were added to the proteoliposomes before the passage of the proteoliposomes through the Sephadex G-75 columns and, then, the transport was started as described above. In all the samples the transport reaction was stopped after 10 min, adding 20 μM mersalyl. Percent residual activity with respect to the control (92 ± 16 nmol/mg protein) is reported. The values are means \pm S.D. from three experiments.

(Fig. 4A–C) or Na^+ (Fig. 5A–C) concentration in the absence or presence of the reagents was studied. The data, analysed in double reciprocal (Lineweaver–Burk) plots was interpolated by straight lines corresponding to the controls (without reagents) or to different reagent concentrations, with common intersections close to the abscissa. These patterns demonstrate non-competitive inhibition. The half saturation constants of the transporter for the inhibitors (K_i) were derived from the plots. The values of the K_i calculated from Fig. 4 were 0.85 ± 0.45 , 1.1 ± 0.15 or 3.3 ± 0.50 μM for HgCl_2 , methylmercury and mersalyl, respectively. The values of the K_i calculated from Fig. 5 were 1.6 ± 0.10 , 1.7 ± 0.19 or 1.9 ± 0.10 μM for HgCl_2 , methylmercury and mersalyl, respectively. These values are similar to the IC_{50} , as in the case of non-competitive inhibition. However, in the case of covalent inhibition a non-competitive pattern can be observed also if the inhibitors interact with the substrate binding site [38]. This can be discriminated by protection experiments. If the active and inhibitory sites overlap, the addition of the substrate before the inhibitor causes a reduction of the inhibition. To clarify this point the proteoliposomes were incubated with the glutamine or Na^+ at different concentrations before the addition of methylmercury. The concentration of the reagent was kept relatively low (close to the IC_{50}) to emphasize the possible protection effect. The residual activity of the transporter in the presence of methylmercury, that was 38% (62% inhibition) of the control, was virtually not influenced by the pre-incubation with the substrates, independently on the concentrations (Fig. 6). Very similar results were found in the case of HgCl_2 and mersalyl (not shown). This data confirmed that the reagents bound to a site(s) of the protein which was different from the substrate binding site.

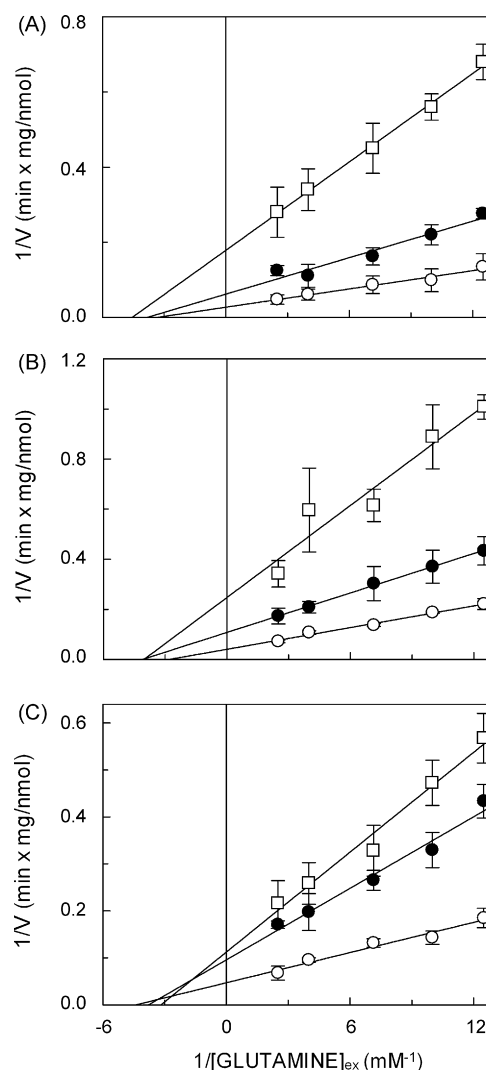


Fig. 4. Kinetic analysis of the inhibition of the reconstituted transporter by mercuric reagents at variable glutamine concentrations. Transport was measured adding [^3H]glutamine at the indicated concentrations and 50 mM Na-gluconate at time zero to proteoliposomes containing 30 mM glutamine in the absence (○) or in the presence of 1.5 μM (●) or 3.0 μM (□) HgCl_2 (A), or in the presence of 2.0 μM (●) or 5.0 μM (□) methylmercury (B) or in the presence of 3.0 μM (●) or 5.0 μM (□) mersalyl (C). The transport reaction was stopped at 10 min, adding 20 μM mersalyl. The experimental data were plotted according to Lineweaver–Burk as reciprocal transport rate vs reciprocal glutamine concentrations. The values are means \pm S.D. from three experiments.

3.3. Transport of mercuric compounds

It was reported that the amino acid transport systems LAT1, LAT2 and B $^{0,+}$, as well as organic anion transporters are able to transport thiol conjugates of methylmercury, especially as cysteine-S-conjugates [2,5,7–9]. To test whether ASCT2 could transport the mercuric reagents as conjugates with cysteine or N-acetylcysteine, the efflux procedure was used. In these experiments the proteoliposomes were prelabeled by transporter-mediated exchange equilibration (see Section 2). After this procedure the proteoliposomes contained internal 30 mM [^3H]glutamine. The addition of unlabeled 1.0 mM glutamine or cysteine caused efflux of the internal radioactivity due to the [^3H]glutamine $_{\text{in}}$ /glutamine $_{\text{out}}$ homologous or [^3H]glutamine $_{\text{in}}$ /cysteine $_{\text{out}}$ heterologous antiport; on the contrary nearly no variations of internal radioactivity were observed after the addition of 1.0 mM N-acetylcysteine, methylmercury, methylmercury-cysteine or methylmercury-N-acetylcysteine conjugates. Similar results were

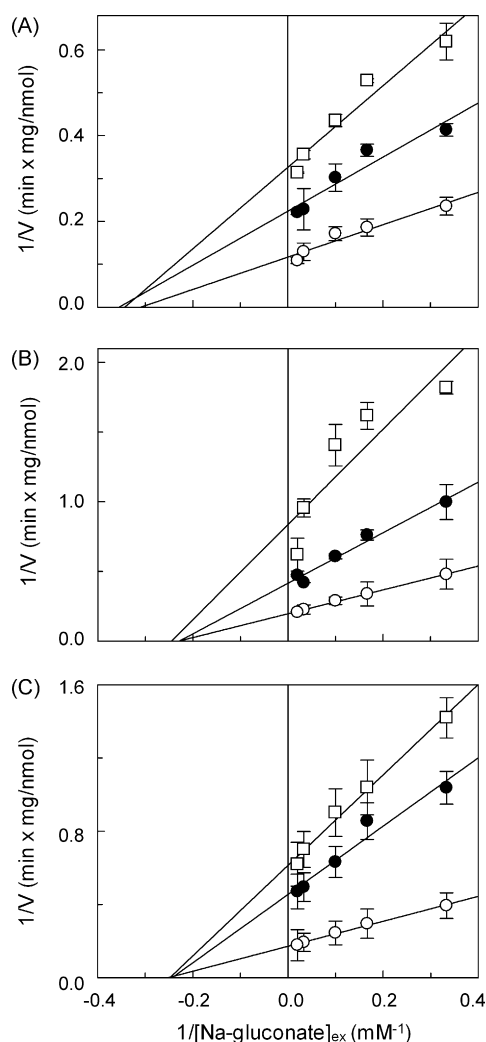


Fig. 5. Kinetic analysis of the inhibition of the reconstituted transporter by mercuric reagents at variable Na^+ concentrations. Transport was measured adding 0.1 mM [^3H]glutamine and the indicated concentrations of Na-gluconate at time zero to proteoliposomes containing 30 mM glutamine in the absence (\circ) or in the presence of 1.5 μM (\bullet) or 3.0 μM (\square) HgCl_2 (A), or in the presence of 2.0 μM (\bullet) or 5.0 μM (\square) methylmercury (B) or in the presence of 3.0 μM (\bullet) or 5.0 μM (\square) mersalyl (C). The transport reaction was stopped at 10 min, adding 20 μM mersalyl. The experimental data were plotted according to Lineweaver-Burk as reciprocal transport rate vs reciprocal Na-gluconate concentrations. The values are means \pm S.D. from three experiments.

observed after the addition of the unconjugated and conjugated Hg^{2+} and mersalyl (not shown). The small decrease of intraliposomal radioactivity was comparable to that found in the absence of external substrate (Fig. 7A). This data indicated that cysteine was transported by the reconstituted ASCT2, as previously shown [27], whereas N-acetylcysteine and all the conjugates did not. In another experiment the uptake of [^3H]glutamine into proteoliposomes containing the same compounds externally added in the previous experiment was studied. To compare the data concerning the methylmercury conjugated with that of the control, the concentration of intraliposomal glutamine was kept at the same concentration (1.0 mM) of the compounds. Thus, the uptake of [^3H]glutamine in the control proteoliposomes was lower than that measured in the experiments of Fig. 1 in which the intraliposomal glutamine concentration was 30 mM. The accumulation of labeled glutamine in the proteoliposomes in the presence of each of the compounds was much lower than the control and comparable or only slightly higher (in the case of cysteine) in respect to that found in the absence of intraliposomal substrate, indicating that none of

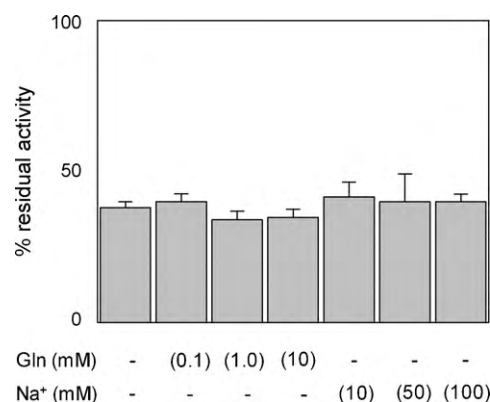


Fig. 6. Influence of substrate on the inhibition of the reconstituted transporter by mercuric reagents. Reconstituted proteoliposomes were treated as described in the figure before the transport assay: where indicated, glutamine or Na-gluconate at the concentrations (mM) in brackets were added to proteoliposome samples 1 min before 3.0 μM methylmercury. All samples were then passed through Sephadex G-75 columns to remove the unreacted inhibitor and substrates; transport was started adding 0.1 mM [^3H]glutamine and stopped after 30 min adding 20 μM mersalyl. Percent residual activity with respect to the control (167 ± 27 nmol/mg protein) is reported. The values are means \pm S.D. from three experiments.

the compounds was transported from inside to outside (Fig. 7B). Similar data were found in the presence of intraliposomal unconjugated and conjugated Hg^{2+} and mersalyl (not shown). The data also correlates with the previous finding [27] that

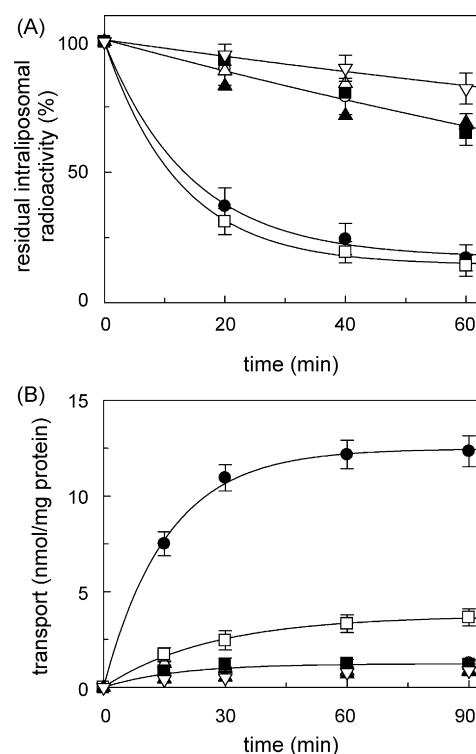


Fig. 7. Time course of [^3H]glutamine efflux and uptake in proteoliposomes mediated by the reconstituted transporter in the presence of mercuric conjugates as counter-substrates. (A) Efflux or (B) uptake of [^3H]glutamine in proteoliposomes was measured in the presence of: (A) external 50 mM Na-gluconate and 1.0 mM glutamine (\bullet), 1.0 mM cysteine (\square), 1.0 mM N-acetylcysteine (\blacksquare), 1.0 mM methylmercury (\triangle), 1.0 mM methylmercury-cysteine (\blacktriangle), 1.0 mM methylmercury-N-acetylcysteine (∇) or buffer alone (\circ); (B) external 50 mM Na-gluconate and internal 1.0 mM glutamine (\bullet), 1.0 mM cysteine (\square), 1.0 mM N-acetylcysteine (\blacksquare), 1.0 mM methylmercury (\triangle), 1.0 mM methylmercury-cysteine (\blacktriangle), 1.0 mM methylmercury-N-acetylcysteine (∇) or buffer alone (\circ). The transport reaction was stopped at the indicated times, adding 20 μM mersalyl. The values are means \pm S.D. from three experiments.

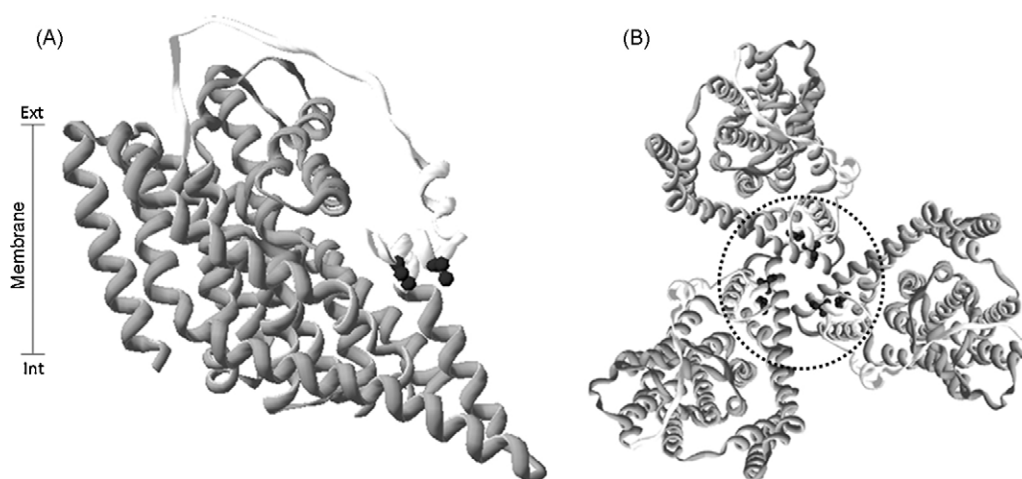


Fig. 8. Homology structural model of rat ASCT2. (A) Ribbon diagram viewing the transporter from the lateral side; the membrane is indicated by a black line with external and internal sides. (B) Ribbon diagram viewing the hypothetical trimeric form of the transporter from the top (extracellular side); the basin protruding towards the extracellular side is indicated by a dotted circle. Cys-207 and Cys-210 are in dark grey. The large loop protruding towards the extracellular environment is in white. The homology model has been represented using the molecular program SpdbViewer 4.01.

cysteine is transported from outside to inside (Fig. 7A) but not vice versa (Fig. 7B).

3.4. Prediction of the inhibition site of the ASCT2 transporter

It was previously reported that, probably, cysteine SH group(s) of the reconstituted ASCT2 transporter are accessible from the external side of the proteoliposomes, which corresponds to the extracytoplasmic face of the transporter [27]. These functional groups could be involved in the mechanism of inhibition by mercurials. To predict which of the Cys residues of the ASCT2 transporter could be involved in mercurial binding, a bioinformatic approach was used. As it was previously reported, ASCT2 belongs to the transporter family SLC1. This family includes also ASCT1 and EAAT1 [39], which are homologues of the glutamate transporter Glt_{ph}, whose structure was resolved by X-ray crystallography [33]. In particular it was reported that ASCT1 has the same consensus sequence of Glt_{ph} and, hence, it may have the same structural fold. As the human ASCT2 and ASCT1 [40], the rat ASCT2 sequence shares 56% identity with the rat ASCT1 and the same consensus sequences of Glt_{ph} (not shown) confirming that ASCT2 belongs to the same transporter family. Thus the ASCT2 sequence has been aligned with the Glt_{ph} and the alignment has been used to construct the homology model (Fig. 8). The structural model of ASCT2 showed properties similar to those of Glt_{ph} structure [33,41]. The protein appears as a wedge with the wide end in contact with the membrane (Fig. 8A). The tips of three subunits probably come together, as in Glt_{ph}, forming a trimeric structure. This structure forms, in the middle, a basin exposed towards the extracellular environment (Fig. 8B). Interestingly, ASCT2 contains a CXXC motif which is typical of metal binding sites in proteins [42]. The two cys residues (Cys-207 and Cys-210) of the motif, highlighted in dark grey (Fig. 8A and B), are located at the end of a large hydrophilic loop (highlighted in white) which is exposed in the basin towards the external side of the protein. The two Cys are surrounded by four hydrophobic residues, Leu-106, Val-110, Met-225 and Met-227 (not shown).

4. Discussion

In the present study, the proteoliposome system has been used to study the interaction of methylmercury and other mercuric reagents with the reconstituted glutamine/amino acid transporter, which was previously identified with ASCT2 [27,28]. HgCl₂ and

methylmercury are the most common forms of mercury to which humans are exposed [4,6,7]; mersalyl has been used as a prototypal hydrophilic mercury compound which is frequently used as a specific reagent for Cys residues of proteins [35]. Preliminary studies performed with hydrophilic SH reagents, suggested that the reconstituted transporter contains thiol groups of cysteine exposed towards the extraliposomal compartment, which corresponds to the extracellular environment; these data correlated well with the predicted hydropathy profile of ASCT2 [27,40]. In this profile 2 Cys residues are located in a large hydrophilic loop exposed towards the extracellular environment (not shown and see Ref. [27]). Thus, it was expected that the mercuric reagents could interact with one or more of the exposed Cys residue(s) of the transporter. Indeed, the experimental data described in this work showed that HgCl₂, methylmercury and mersalyl interact with the transporter leading to complete inactivation of its transport function. By experiments based on the pre-incubation of the proteoliposomes with the reagents and subsequent removal of the unreacted compounds it was demonstrated that the effect was due to covalent binding of the mercuric reagents with one or more Cys residue. The kinetic analysis of the inhibition showed a non-competitive behavior of the reagents which indicated that the site of action should be different from the substrate binding site. This hypothesis was confirmed by the finding that the IC₅₀ and the K_i values of the transporter for each of the compounds were similar and no protection of the inhibition was observed neither by glutamine nor by Na⁺. To better evaluate the relationships between the inhibition by mercuric reagents and the structure of the transporter, the homology structural model of the rat ASCT2 was built using bioinformatics. In this model the positions of at least two Cys residues correlate well with the experimental data. The residues Cys-207 and Cys-210, which belong to a CXXC metal binding motif, are located at the end of a large hydrophilic loop of the transporter. The transport protein may have a trimeric form as described for the Glt_{ph} [42], with a concave aqueous basin in the middle facing towards the extracellular side (Fig. 8B). The two Cys residues are located approximately in the middle of the basin in the proximity of four hydrophobic residues. This may explain the finding that the transporter shows higher affinity for methylmercury in respect to the more hydrophilic mersalyl which, in addition, exerts stronger inhibition at longer times of incubation (see Section 3 and Fig. 3). The involvement of the CXXC motif in the inhibition is in agreement with the higher affinity of the transporter for HgCl₂

(Hg^{2+}), which can react with two vicinal SH, than to methylmercury and mersalyl, which can react with only one Cys [35]. Among other metal cations which are known to react with vicinal SH groups [35], Cu^{2+} strongly inhibited the reconstituted transporter (IC_{50} , 20 μM) with a non-competitive mechanism; whereas Cd^{2+} and Zn^{2+} were poor inhibitors with $\text{IC}_{50} > 500 \mu\text{M}$ (Oppedisano F. and Indiveri C., unpublished results), indicating that the interaction is specific. A third residue, Cys-223, is present in the proximity of the CXXC motif. It cannot be excluded that this Cys residues may also be involved in the reaction with mercuric reagents. The inhibition of the transport function upon reaction of the mercuric reagents with the Cys residue(s) may be caused by impairment of mobility of the hydrophilic loop and/or other domains of the transporter, which is necessary for the transport cycle.

The implication of the mechanisms of inhibition of the ASCT2 transporter in toxicology may be relevant since this transport system plays important functions in several districts among which the intestine, kidney and nervous tissue [39,40]. The inhibitory effects have been observed at concentrations far below the LD_{50} of mercuric reagents [1]. Thus, the inactivation of the ASCT2 transporter may impair the permeability and the metabolism of tissues and organs in which it is expressed, under conditions in which the mercuric compound is still below the level of acute toxicity. Methylmercury is readily absorbed by the intestine and reaches normally in blood the concentration of 1–8 $\mu\text{g/l}$. Above 200 $\mu\text{g/l}$ (about 1 μM), methylmercury exerts toxic effects [6]. The IC_{50} of the transporter for this reagent is in the same range of the toxicity threshold. At the renal or intestine level the inactivation of ASCT2 may have little influence on overall amino acid adsorption or excretion, due to the antiport mode of functioning. However, it may cause imbalance in the composition of the amino acid pool in the cells with, for example, decrease of cysteine, valine and methionine which are transported by ASCT2 only from the external to the internal environment but not vice versa (see Fig. 7 and Ref. [27]). The inactivation of ASCT2 will have important consequences in the nervous tissue. It was previously reported that methylmercury inhibits the expression of the amino acid transporters SNAT1 and ASCT2 in astrocytes. However, this mechanism could not explain the effects caused at prolonged times of exposure to methylmercury [26]. These effects are explained by the direct inhibition of the transporter, the activity of which is suppressed by 10 μM methylmercury (see Fig. 3), i.e., the concentration used in the previous study [26]. The effects of cysteine and N-acetylcysteine in reversing the inhibition by mercurials correlates well with the use of these antioxidant compounds in mercury detoxification [4]. Differently from other amino acid or anion transporters [2,5,7–9] ASCT2 is not involved in the absorption of mercurials nor as free molecules neither as conjugates.

Acknowledgements

This work was supported by grants from Ministero dell'Università e della Ricerca (PRIN 2006 grant no. 2006054479) and from University of Calabria (ex 60% 2008–2009).

References

- [1] Zalups RK. Molecular interactions with mercury in the kidney. *Pharmacol Rev* 2000;52:113–43.
- [2] Bridges CC, Zalups RK. Molecular and ionic mimicry and the transport of toxic metals. *Toxicol Appl Pharmacol* 2005;204:274–308.
- [3] Newland MC, Paletz EM, Reed MN. Lactational exposure to mercury in experimental models. *Neurotoxicology* 2009;30:160–3.
- [4] Rooney JP. The role of thiols, dithiols, nutritional factors and interacting ligands in the toxicology of mercury. *Toxicology* 2007;234:145–56.
- [5] Hirooka T, Fujiwara Y, Minami Y, Ishii A, Ishigooka M, Shinkai Y. Cell-density-dependent methylmercury susceptibility of cultured human brain microvessel pericytes. *Toxicology In Vitro* 2010;24:835–41.
- [6] ATSDR: Agency for Toxic Substance and Disease Registry. Toxicological profile for mercury. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Services, Centers for Disease Control; 2003.
- [7] Bridges CC, Zalups RK. System $\text{b}^{0,+}$ and the transport of thiol-S-conjugates of methylmercury. *J Pharmacol Exp Ther* 2006;319:948–56.
- [8] Yin Z, Jiang H, Syversen T, Rocha JB, Farina M, Aschner M. The methylmercury-L-cysteine conjugate is a substrate for the L-type large neutral amino acid transporter. *J Neurochem* 2008;107:1083–90.
- [9] Heggland I, Kaur P, Syversen T. Uptake and efflux of methylmercury in vitro: comparison of transport mechanisms in C6, B35 and RBE4 cells. *Toxicol In Vitro* 2009;23:1020–7.
- [10] Ritzhaupt A, Wood IS, Ellis A, Hosie KB, Shirazi-Beechey SP. Identification and characterization of a monocarboxylate transporter (MCT1) in pig and human colon: its potential to transport L-lactate as well as butyrate. *J Physiol* 1998;513:719–32.
- [11] Schweri MM. Mercuric chloride and p-chloromercuriphenylsulfonate exert a biphasic effect on the binding of the stimulant [3H]methylphenidate to the dopamine transporter. *Synapse* 1994;16:188–94.
- [12] Zhang ZH, Solomon AK. Effect of pCMBS on anion transport in human red cell membranes. *Biochim Biophys Acta* 1992;1106:31–9.
- [13] Zeidel ML, Albalak A, Grossman E, Carruthers A. Role of glucose carrier in human erythrocyte water permeability. *Biochemistry* 1992;31:589–96.
- [14] Pochini L, Oppedisano F, Indiveri C. Reconstitution into liposomes and functional characterization of the carnitine transporter from renal cell plasma membrane. *Biochim Biophys Acta* 2004;1661:78–86.
- [15] Oppedisano F, Indiveri C. Reconstitution into liposomes of the B^0 -like glutamine-neutral amino acid transporter from renal cell plasma membrane. *Biochim Biophys Acta* 2008;1778:2258–65.
- [16] Palmieri F, Indiveri C, Bisaccia F, Iacobazzi V. Mitochondrial metabolite carrier proteins: purification, reconstitution, and transport studies. *Methods Enzymol* 1995;260:349–69.
- [17] Palmieri F, Indiveri C, Bisaccia F, Kramer R. Functional properties of purified and reconstituted mitochondrial metabolite carriers. *J Bioenerg Biomembr* 1993;25:525–35.
- [18] Verkman AS. Water channels in cell membranes. *Annu Rev Physiol* 1992;54:97–108.
- [19] Anner BM, Moosmayer M. Mercury inhibits Na-K-ATPase primarily at the cytoplasmic side. *Am J Physiol* 1992;262:F843–8.
- [20] Anner BM, Moosmayer M, Imesch E. Mercury blocks Na-K-ATPase by a ligand-dependent and reversible mechanism. *Am J Physiol* 1992;262:F830–6.
- [21] Murata K, Mitsuoka K, Hirai T, Walz T, Agre P, Heymann JB, et al. Structural determinants of water permeation through aquaporin-1. *Nature* 2000;407:599–605.
- [22] Capobianco L, Bisaccia F, Mazzeo M, Palmieri F. The mitochondrial oxoglutarate carrier: sulfhydryl reagents bind to cysteine-184, and this interaction is enhanced by substrate binding. *Biochemistry* 1996;35:8974–80.
- [23] Wellner M, Monden I, Keller K. The role of cysteine residues in glucose-transporter-GLUT1-mediated transport and transport inhibition. *Biochem J* 1994;299:813–7.
- [24] Tonazzi A, Indiveri C. Chemical modification of the mitochondrial ornithine/citrulline carrier by SH reagents: effects on the transport activity and transition from carrier to pore-like function. *Biochim Biophys Acta* 2003;1611:123–30.
- [25] Broer S. Amino acid transport across mammalian intestinal and renal epithelia. *Physiol Rev* 2008;88:249–86.
- [26] Yin Z, Milatovic D, Aschner JL, Syversen T, Rocha JB, Souza DO, et al. Methylmercury induces oxidative injury, alterations in permeability and glutamine transport in cultured astrocytes. *Brain Res* 2007;1131:1–10.
- [27] Oppedisano F, Pochini L, Galluccio M, Cavarelli M, Indiveri C. Reconstitution into liposomes of the glutamine/amino acid transporter from renal cell plasma membrane: functional characterization, kinetics and activation by nucleotides. *Biochim Biophys Acta* 2004;1667:122–31.
- [28] Oppedisano F, Pochini L, Galluccio M, Indiveri C. The glutamine/amino acid transporter (ASCT2) reconstituted in liposomes: transport mechanism, regulation by ATP and characterization of the glutamine/glutamate antiport. *Biochim Biophys Acta* 2007;1768:291–8.
- [29] Biber J, Stieger B, Haase W, Murer H. A high yield preparation for rat kidney brush border membranes. Different behaviour of lysosomal markers. *Biochim Biophys Acta* 1981;647:169–76.
- [30] Kramer R, Heberger C. Functional reconstitution of carrier proteins by removal of detergent with a hydrophobic ion exchange column. *Biochim Biophys Acta* 1986;863:289–96.
- [31] Palmieri F, Klingenberg M. Direct methods for measuring metabolite transport and distribution in mitochondria. *Methods Enzymol* 1979;56:279–301.
- [32] Dulle J, Grieve PA. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Anal Biochem* 1975;64:136–41.
- [33] Yernool D, Boudker O, Jin Y, Gouaux E. Structure of a glutamate transporter homologue from *Pyrococcus horikoshii*. *Nature* 2004;431:811–8.
- [34] Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* 1993;234:779–815.
- [35] van Iwaarden PR, Driessen AJ, Konings WN. What we can learn from the effects of thiol reagents on transport proteins. *Biochim Biophys Acta* 1992;1113:161–70.

- [36] Tonazzi A, Giangregorio N, Palmieri F, Indiveri C. Relationships of Cysteine and Lysine residues with the substrate binding site of the mitochondrial ornithine/citrulline carrier: an inhibition kinetic approach combined with the analysis of the homology structural model. *Biochim Biophys Acta* 2005;1718:53–60.
- [37] Pochini L, Galluccio M, Scumaci D, Giangregorio N, Tonazzi A, Palmieri F, et al. Interaction of beta-lactam antibiotics with the mitochondrial carnitine/acyl-carnitine transporter. *Chem Biol Interact* 2008;173:187–94.
- [38] Webb JL. Enzyme and metabolic inhibitors. New York and London: Academic Press; 1966.
- [39] Kanai Y, Hediger MA. The glutamate/neutral amino acid transporter family SLC1: molecular physiological and pharmacological aspects. *Pflugers Arch* 2004;447:469–79.
- [40] Utsunomiya-Tate N, Endou H, Kanai Y. Cloning and functional characterization of a system ASC-like Na⁺-dependent neutral amino acid transporter. *J Biol Chem* 1996;271:14883–90.
- [41] Reyes N, Ginter C, Boudker O. Transport mechanism of a bacterial homologue of glutamate transporters. *Nature* 2009;462:880–5.
- [42] Vats N, Lee SF. Characterization of a copper-transport operon, copYAZ, from *Streptococcus mutans*. *Microbiology* 2001;147:653–62.